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13. ABSTRACT *(Maximum 200*

The proposal of my grant is to investigate the biological significance and mechanism of IGFBP-3 as well as identification and characterization of the IGFBP-3 receptor in human breast cancer cells. As a first year task, I initiated the identification of the putative IGFBP-3 receptor in human breast cancer cells by employing the yeast two-hybrid system. To date this approach has been successful using total Hs578T breast cancer cell mRNA for generation of a cDNA library, and a cDNA encoding an IGFBP-3 fragment (IGFBP-3^{88-148}) as a "bait". Preliminary screening of transformant colonies was successful, yielding several hundred positive interaction clones. Testing is currently underway for interaction specificity, after which individual cDNAs that prove interesting will be isolated and further characterized. Proceeding with our research for IGF-independent action of IGFBP-3 in breast cancer cells, I have identified new low-affinity IGFBPs (IGFBP-7 and -8). These proteins show similar characteristics in their biological actions, which are comparable to those of IGFBP-3 in terms of breast cancer cell growth inhibition. Therefore, in combination with the IGFBP-3 receptor studies I am also investigating these new potent growth inhibitors of breast cancer cells for inclusion as members of IGFBP superfamily.

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Joseph Oh 10/29/97

PI - Signature Date

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I. INTRODUCTION.

The insulin-like growth factor binding proteins (IGFBPs) are members of the IGF signaling system, along with the ligands (insulin, IGF-I and IGF-II) and a family of transmembrane receptors (the insulin, type 1 IGF and type 2 IGF receptors) (1-9). The human IGFBP family consists of at least seven proteins, designated as IGFBP-1,-2, -3, -4, -5 -6 and-7 (10-12) (Table 1). IGFBPs 1-6 bind IGF-I and IGF-II with high affinity and serve to transport the IGFs, prolong their half-lives, and modulate their proliferative and anabolic effects on target cells. The molecular mechanisms involved in the interaction of the IGFBPs with the IGFs and their receptors remain unclear, but these molecules appear, at least, to regulate the availability of free IGFs for interaction with IGF receptors (10, 11). Recently, we have identified mac25 as IGFBP-7, which binds IGFs with lower affinity than do the other IGFBPs, and which appears to be a low-affinity member of the IGFBP family (Appendix #1).

Recent evidence indicates that some IGFBPs may also have IGF-independent effects. A growing body of data has demonstrated that IGFBP-3 is an important growth-suppressing factor in various cell systems, an effect which is mediated in an IGF-independent manner. In this project, I have proposed investigation of the biological significance and mechanism of this IGF-independent action of IGFBP-3 as well as identification and characterization of an IGFBP-3 receptor in human breast cancer cells. As a first year task, I initiated the identification of the putative IGFBP-3 receptor in human breast cancer cells by employing a yeast two-hybrid system. In addition, I have generated IGFBP-3 fragments using a baculovirus expression system for characterization of structure-function aspects of IGFBP-3 actions.

II. BODY.

1. Yeast Two-Hybrid Screen for the IGFBP-3 Receptor in Hs578T Human Breast Cancer Cells

I had initially proposed to employ expression cloning by use of lambda expression libraries for identification of an IGFBP-3 receptor. The expression library, consisting of Hs578T-derived cDNAs cloned into the lgt11 expression vector, was generated and screened using [^{125}I]IGFBP-3 or polyclonal IGFBP-3 receptor antibodies. However, I have faced technical difficulties due to the nature of the IGFBP-3 protein, specifically its ability to bind proteins in a non-specific manner under these experimental conditions. As an alternative approach, yeast two-hybrid screening was employed to identify IGFBP-3 interacting proteins (13). Previous data had indicated that binding sites for a putative IGFBP-3 receptor resides in the mid-region of the IGFBP-3 molecule. A fragment containing this IGFBP-3 mid-region (IGFBP-3$^{88\text{-}148}$) binds to Hs578T cell surfaces with high affinity, similar to that of full-length IGFBP-3 (Figure 1). Using this Intermediate fragment as the "bait", we are screening a cDNA library made from an Hs578T mRNA preparation to pull out the putative IGFBP-3 receptor.

The bait plasmid was constructed using the pBTM116 vector, containing the coding sequences of the TRP1 gene for tryptophan synthesis in yeast, and the LexA gene. Into this vector, the DNA sequence coding for amino acids 88-148 of IGFBP-3 and a FLAG tag was fused inframe behind the LexA coding sequence, creating a LexA : IGFBP-3$^{88\text{-}148}$/FLAG fusion gene. This bait plasmid was transformed into the yeast strain L40 and grown on trp-lacking media. Two reporter constructs are maintained in the L40 yeast strain (14). The first, LYS2 : $(lexAop)_4$-HIS3, is fused at the yeast LYS2 locus and will confer histidine prototrophy upon activation by LexA. The second, URA3 : $(lexAop)_8$-lacZ, is fused at the yeast URA3 locus and will result in a blue-colored yeast colony upon activation of the LexA operators (This strain is maintained on lys- and ura-lacking media to guard against the loss of these reporters).

The Hs578T cDNA library was cloned into the pGAD10 vector (Clonetech), which contains the coding sequences of the LEU2 gene for leucine synthesis in yeast, and the GAL4 activation domain (GAL4AD). Individual cDNAs were inserted ahead of the GAL4 coding

sequence, creating a cDNA : GAL4AD fusion library. This library was subsequently introduced into the L40/IGFBP-3^{88-148} yeast strain. Transformant colonies were screened using a β-galactosidase assay to check for activation of the lacZ gene by the interaction of LexA : IGFBP-3^{88-148} (conferring binding to the LexA operators) with the cDNA::GAL4AD (conferring activation). Blue colonies, containing cDNAs that potentially are interacting with the IGFBP-3 fragment will then be "cured" of the bait plasmid by successive culturing on trp-containing media and tested by culturing on trp+ and trp- media to confirm bait plasmid loss. Specificity of the interactions will be tested by mating with the original yeast bait strain, and with a strain containing LexA::lamin (commonly used to detect nonspecific interactions), followed by a β-galactosidase test. Colonies which remain positive (blue) with the original bait, but are negative with lamin will be further characterized. Plasmid DNA from these yeast colonies, now containing only the cDNA : GAL4AD fusion plasmids of specific interest, will be prepared and each subsequently transformed into *E. coli*, where the individual cDNAs will be isolated and characterized to potentially identify the IGFBP-3 receptor.

2. Identification of a New Low-Affinity Member of IGFBP Family

Recently, my laboratory has identified mac25 as IGFBP-7 using baculovirus expressed recombinant human mac25 and polyclonal antibodies specific for human mac25 (appendices # 1 and 2). The mac25 gene, residing on chromosome 4q12 and encoding a pre-protein of 277 amino acids, was originally cloned from leptomeningial cells and subsequently re-isolated through differential display as a sequence preferentially expressed in senescent human mammary epithelial cells (12). The deduced amino acid sequence of the mac25 propeptide shows an overall 40-45% similarity and 20-25% identity to IGFBPs. Furthermore, mac25 contains the common IGFBP motif (GCGCCXXC) at the NH_2-terminus, in a region containing a cluster of 12 conserved cysteines, of which 11 are found in mac25.

The 1.1 kb mac25 mRNA was detected in a wide range of normal human tissues, with decreased expression in breast, prostate, colon and lung cancer cell lines (Appendix #1). The 31 kDa IGFBP-7 protein was detected in the conditioned media of Hs578T breast cancer cells, as well as in normal human urine, cerebrospinal fluid and amniotic fluid (Appendix #2).

Binding of ^{125}I-IGF-I and ^{125}I-IGF-II to rh-mac25 was demonstrated by western ligand blotting after non-denaturing polyacrylamide gel electrophoresis and by affinity cross-linking. Compared to IGFBP-3, rh-mac25 showed at least a 5-6 fold lower affinity for IGF-I and 20-25 fold lower affinity for IGF-II, indicating that IGFBP-7 constitutes a low affinity member of IGFBP family (Appendix #1).

To extend our understanding of the IGF-independent action of IGFBPs, the biological action of IGFBP-7 was investigated in human breast cancer cells. Using baculovirus expressed IGFBP-7 (IGFBP-7^{bac}) and polyclonal antibodies specific for IGFBP-7, I have found that expression of both IGFBP-7 mRNA and protein is up-regulated by TGF-b in Hs578T breast cancer cells. Intriguingly, treatment with IGFBP-7^{bac} resulted in inhibition of DNA synthesis and cell proliferation in a dose-dependent manner in human breast cancer cells, even in the absence of IGF peptides (Figure 3). In addition, IGFBP-7 mRNA was down-regulated in human breast cancer cells and up-regulated in normal, growing mammary epithelial cells by RA. It is clear that while IGFBP-7 constitutes a low affinity member of the IGFBP family, it primarily functions as a modulator of cell growth in an IGF-independent manner, similar to actions observed with IGFBP-3 in breast cancer cells. Therefore, studies on the mechanisms of action and signaling pathways used by IGFBP-7 will provide insight into the IGF-independent actions of the classical high affinity IGFBPs, especially IGFBP-3.

3. Synthesis of IGFBP-3 Fragments

Previous studies have demonstrated that the effect of IGFBP-3 on IGF-induced mitogenic action can be modulated by IGFBP-3 proteases. It has been demonstrated that IGFBP-3 proteases appear to enhance mitogenic action of IGFs; proteolysis of IGFBP-3 results in significantly reduced affinity for IGF peptides, thereby facilitating IGF binding to its receptors. Several proteases have been identified in human breast cancer and normal mammary epithelial cells (15-20); some proteases, such as cathepsin D, prostate specific antigen (PSA) have been proposed as prognosis factors because of enhanced expression in breast cancer cells. Interestingly, these proteases, including plasmin (plasminogen activator), are capable of proteolyzing the IGFBP-3

molecule. Therefore, it can be speculated that these proteases can modulate not only the IGFBP-3 effect on IGF binding to IGF receptors, but also IGFBP-3 binding to the IGFBP-3 receptor and its subsequent biological action. IGFBP-3 mutants and proteolyzed fragments of IGFBP-3 will be employed to identify the binding site on IGFBP-3 for the IGFBP-3 receptor as well as the biological significance of IGFBP-3 proteases and IGFBP-3 as a heparin binding protein in the human mammary system.

As a first step to investigate structure-function aspects of IGFBP-3 actions, six different IGFBP-3 fragments were constructed and expressed using a baculovirus expression system (Appendix #3). Based on the known PSA cleavage sites within IGFBP-3 and the predicted plasmin cleavage sites, an N-terminal IGFBP-$3^{1\text{-}97}$ fragment and a C-terminal IGFBP-$3^{98\text{-}264}$ fragment were expressed. By stepwise trucation from the C-terminal end, several C-terminal fragments, such as IGFBP-$3^{98\text{-}232}$, IGFBP-$3^{98\text{-}206}$, IGFBP-$3^{98\text{-}179}$, and IGFBP-$3^{98\text{-}159}$, were further created. Using the available monoclonal anti-IGFBP-3 antibodies, these IGFBP-3 fragments were characterized as derived from either the N-or C-terminal functional domains of IGFBP-3.

The IGFBP-3 fragments will be powerful tools for elucidation of the mechanisms involved in cell surface association of IGFBP-3, either through its receptor or proteoglycans, as well as the biological action of IGFBP-3 as a member of the family of heparin binding proteins as proposed in the present studies. In addition, these fragments can be further applied to investigate: 1) structure-function aspects of IGFBP-3 actions; 2) heparin binding-mediated events of IGFBP-3; and 3) biological significance of IGFBP-3 specific proteases in human mammary cells.

III. CONCLUSIONS.

We have developed the necessary reagents, including IGFBP-3 fragments, a new IGFBP species (IGFBP-7) and antibodies for those proteins, and established a functional system in human breast cancer cells. These studies will provide insights into the multiple actions of IGFBP-3, both IGF-dependent and IGF-independent. Depending on the presence of IGF peptide or the functional status of receptors, IGFBP-3 can exert biological actions either through its own receptor (IGF-independent) or by modulating IGF binding to IGF receptors (IGF-dependent), either of which may provide a mechanism for IGFBP-3 as an anti-proliferation factor in the human mammary system.

A fuller understanding of the IGF-independent action of IGFBPs will allow us to understand how the growth of neoplastic cells can be modulated by the IGF/IGFBP system, and how other growth factors or pharmacological agents can interface with this system. Potentially, this will allow us to develop IGFBP agonists that would provide a new strategy for the endocrine therapy of breast cancer.

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V. APPENDICES

1. Table 1
2. Figure 1
3. Figure 2
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7. Apendix #3

Table 1. Structural characteristics of the human IGFBPs

IGFBP	Molecular weight	Number of amino acids	Number of cysteines	N-linked glycosylation	Chromosomal localization	mRNA size (kb)
High affinity IGFBPs						
IGFBP-1	25,271	234	18	No	7p	1.6
IGFBP-2	31,355	289	18	No	2q	1.5
IGFBP-3	28,717	264	18	Yes	7p	2.4
IGFBP-4	25,957	237	20	Yes	17q	1.7
IGFBP-5	28,553	252	18	No	2q	1.7, 6.0
IGFBP-6	22,847	216	16	No	12	1.1
Low affinity IGFBPs						
IGFBP-7	?	251	18	Yes	4q	1.1

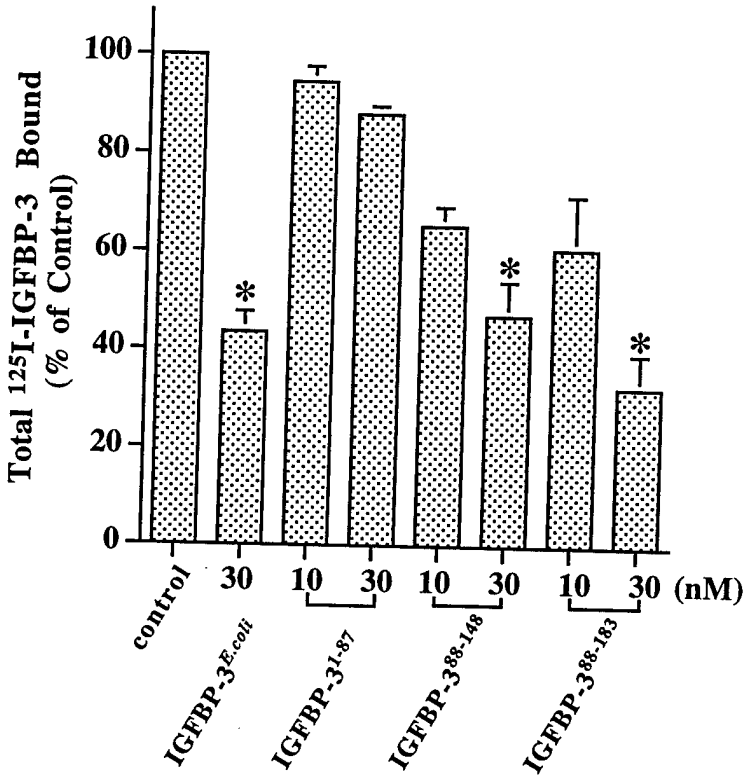
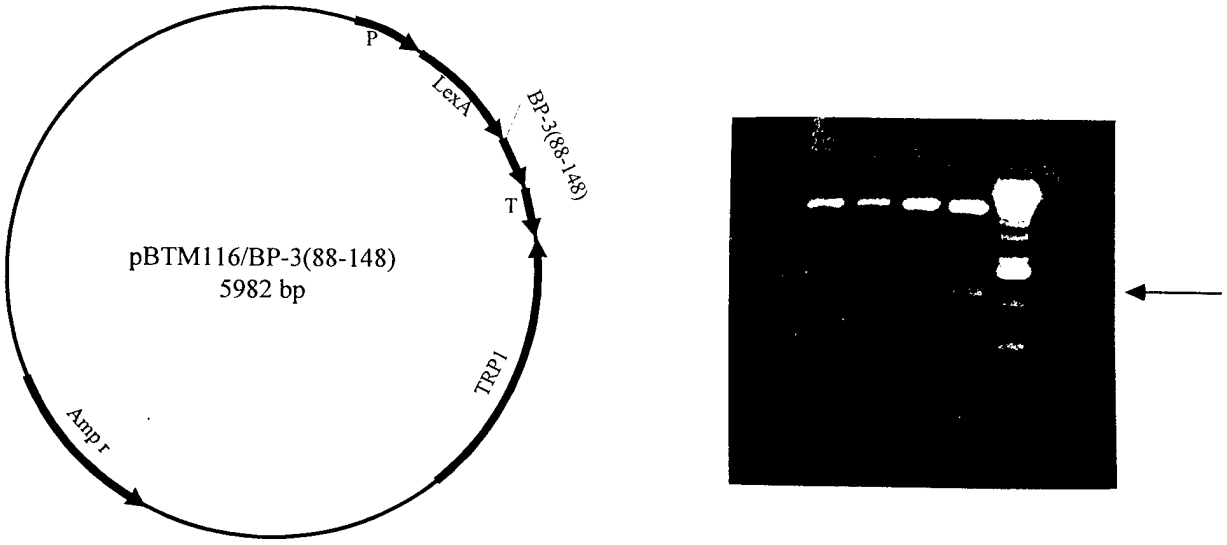


Figure 1. Competitive binding of ^{125}I-IGFBP-3 to Hs578T monolayers by baculovirus expressed recombinant human IGFBP-3 fragments. Experiments were performed using Hs578T human breast cancer monolayers in the absence or presence of indicated concentrations of unlabeled human IGFBP-3 or IGFBP-3 fragments. Statistical significance in comparison with control values is indicated by *$p < 0.01$.

(A)



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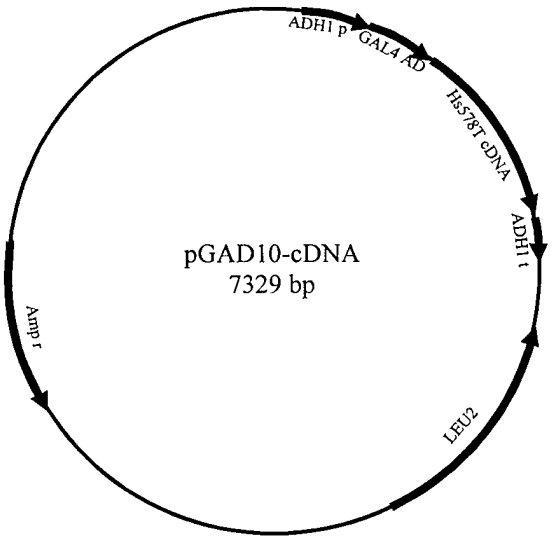

Figure 2. Maps showing construction of the two-hybrid bait and library plasmids for the IGFBP-3 receptor screen. (A) The bait plasmid pBTM116/BP-3(88-148), contains an internal fragment of IGFBP-3. The gel represents DNAs from four individual clones that were digested to excise the BP-3(88-148) fragment, indicating correct construction of the plasmid. Further confirmation was obtained from DNA sequence analysis. Clone #3 was chosen for the two-hybrid screen. (B) pGAD10-cDNA is representative of the library of plasmids containing individual Hs578T cDNAs prepared from total mRNA.

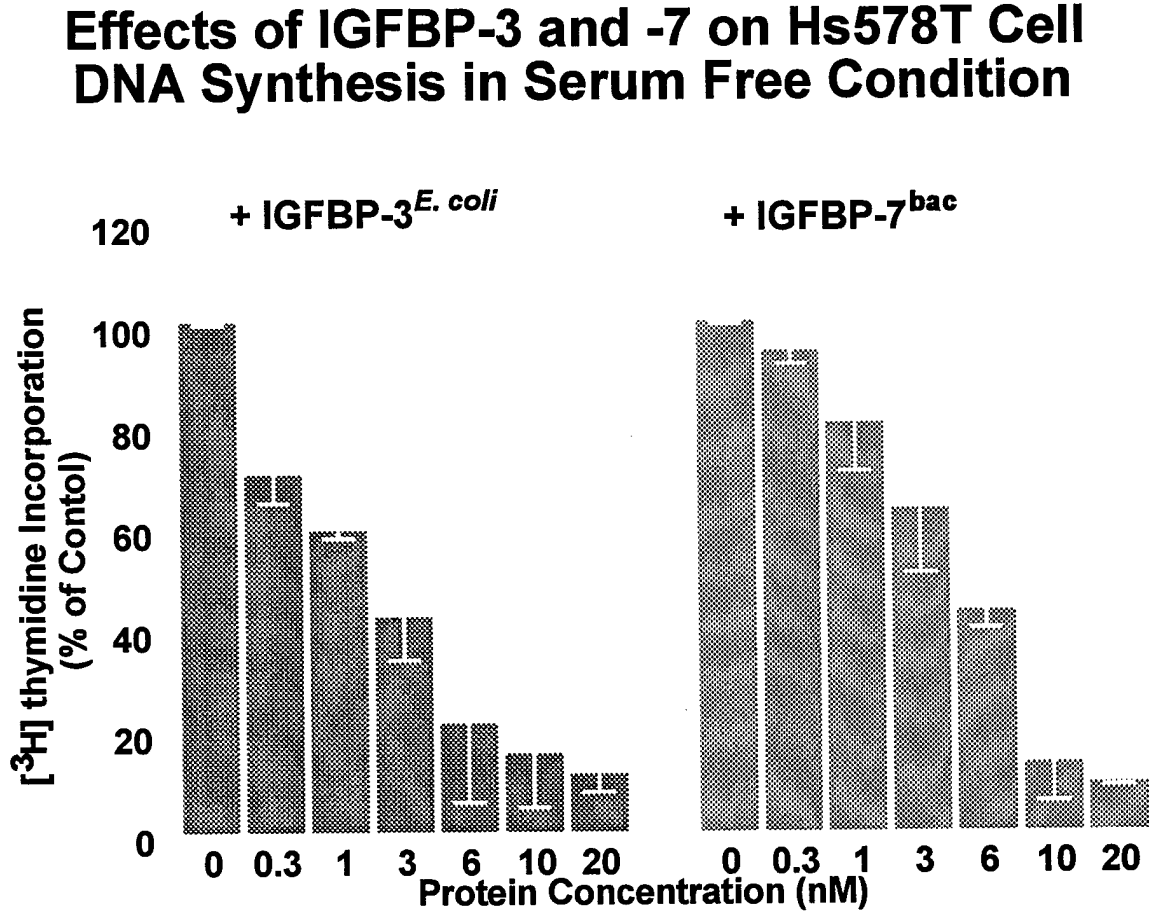

Figure 3. Effect of IGFBP-3 and IGFBP-7 on Hs578T cell DNA synthesis in serum free condition. Cells were grown in 24-multiwell plates until 95% confluent and maintained in serum-free media for 24 h. Cells were then treated with or without various concentrations of IGFBP-3 or IGFBP-7 for 23 h. DNA synthesis was estimated as the actual disintegrations/min of tritiated thymidine incorporation after a 1.5-h pulse of [3H]thymidine.

Communication

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Synthesis and Characterization of Insulin-like Growth Factor-binding Protein (IGFBP)-7

RECOMBINANT HUMAN mac25 PROTEIN SPECIFICALLY BINDS IGF-I AND -II*

(Received for publication, September 5, 1996, and in revised form, September 30, 1996)

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The mac25 cDNA was originally cloned from leptomeningial cells and subsequently reisolated through differential display as a sequence preferentially expressed in senescent human mammary epithelial cells. The deduced amino acid sequence of the human mac25 propeptide shares a 20–25% identity to human insulin-like growth factor-binding proteins (IGFBPs), suggesting that mac25 could be another member of the IGFBP family.

In the present study, we have generated recombinant human mac25 (rh-mac25) in a baculovirus expression system and assessed its affinity for IGFs and have evaluated the pattern of expression of the mac25 gene in human tissues. Binding of ^{125}I-IGF-I and ^{125}I-IGF-II to rh-mac25 was demonstrated by Western ligand blotting after nondenaturing polyacrylamide gel electrophoresis and by affinity cross-linking with as little as 2 nM rh-mac25. Specificity of rh-mac25 binding to ^{125}I-IGFs was demonstrated by competition for rh-mac25 binding with unlabeled IGFs, but not with [QAYLL]IGF-II analog, which has 100-fold less affinity for IGFBPs. In comparison with IGFBP-3, rh-mac25 has at least a 5–6-fold lower affinity for IGF-I and 20–25-fold lower affinity for IGF-II. mac25 mRNA was detectable in a wide range of normal human tissues, with decreased expression in breast, prostate, colon, and lung cancer cell lines.

In conclusion, mac25 specifically binds IGFs and constitutes a new member of the IGFBP family, IGFBP-7. Its wider distribution in normal tissue and lower expression in several cancer cells indicate that IGFBP-7 may function as a growth-suppressing factor, as well as an IGF-binding protein.

The IGFBP[1] family is a critical component of the IGF system. It consists of six distinct proteins, classified as IGFBP-1 to -6, which display no sequence homology to the IGF receptors (1–3). IGFBPs bind IGF peptides with high affinity and regulate the biological activities of the IGFs (4–12). Amino acid sequence analysis has revealed that human IGFBPs show 50–60% similarity and contain 16–18 conserved cysteines at the NH_2- and COOH-terminal regions (1).

The mac25 gene, residing on chromosome 4q12 and encoding a pre-protein of 277 amino acids, has been cloned and sequenced in leptomeningial and mammary epithelial cells (13, 14). mac25 mRNA is preferentially expressed in normal leptomeningial and mammary epithelial cells, compared with their counterpart tumors, suggesting that mac25 may play a role in growth-regulatory pathways that are abrogated in meningiomas and breast carcinoma (13, 14).

Although the mature protein has yet to be identified, the deduced amino acid sequence of the mac25 propeptide shows an overall 40–45% similarity and 20–25% identity to IGFBPs. Furthermore, mac25 contains the common IGFBP motif (GCGCCXXC) at the NH_2 terminus, in a region containing a cluster of 12 conserved cysteines, of which 11 are found in mac25, suggesting that mac25 is another member of the IGFBP family.

In this study, we describe the successful expression of recombinant human mac25, using a baculovirus system, and demonstrate that the 27-kDa mac25 protein specifically binds IGFs, thereby meeting criteria necessary for renaming it IGFBP-7.

EXPERIMENTAL PROCEDURES

Peptides and Proteins—Recombinant human IGF-I was purchased from Bachem (Torrance, CA), and recombinant human IGF-II was provided by Eli Lilly (Indianapolis, IN). Recombinant human IGFBP-3$^{E.\ coli}$, a non-glycosylated 29-kDa core protein which was expressed in *E. coli*, was a generous gift from Celtrix (Santa Clara, CA). Highly specific rabbit polyclonal antibody, αIGFBP-3g1, was raised in our laboratory against glycosylated IGFBP-3 (9). [Gln^6,Ala^7,Tyr^{18},Leu^{19},Leu^{27}]IGF-II ([QAYLL]IGF-II), a synthetic IGF-II analog, was synthesized as described previously (15). Iodination was performed by a modification of the chloramine-T technique, to a specific activity of 350–500 μCi/μg for IGF-I and IGF-II and 100 μCi/μg for IGFBP-3$^{E.\ coli}$ peptides (10). Human multiple tissue Northern blots were purchased from Clontech (Palo Alto, CA).

Cloning and Expression of Recombinant mac25 Protein—Initially, a partial cDNA fragment was generated by reverse transcription-polymerase chain reaction, using RNA isolated from the human breast cancer cell line Hs578T as described previously (16). The primers used for amplification were 5′-primer TGCGAGCAAGGTCCTTCCAT and 3′-primer CACCAGGCAGGAGTTCTGTC (corresponding to nucleotides 479–498 and 628–647 of L198182, GenBank™ sequence). The partial cDNA was subcloned into pGEM-T vector (Promega, Madison, WI) and confirmed by sequencing. An expression cDNA library prepared in ZapExpress (Stratagene, La Jolla, CA) with Hs578T mRNA was screened with the partial cDNA fragment, as described previously (16), and full-length clones were isolated. Full-length sequence was identical to the published mac25 sequence (13).

To express recombinant protein, a FLAG epitope sequence (DYKDDDDK) was added at the COOH terminus by use of polymerase chain reaction. Primers (A) 5′-GGCCATTCAGACCCGGGGTGG (661–682) and (B) 5′-GCCGCCCTCGAGCTACTTCTACTGCTGCTTGTAGTCCCTTTTTTCACTGGCTAT (827–844), which was designed with the FLAG sequence followed by a stop codon and a restriction site for *Xho*I, were used, and the resulting polymerase chain reaction product was digested with *Sma*I and *Xho*I restriction enzymes and ligated into full-length cDNA digested with the above enzymes, to replace the COOH terminus. After sequencing, the FLAG-tagged mac25 cDNA was subcloned into baculovirus expression vector pFASTBAC1 (Life Technologies, Inc.).

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‡ To whom correspondence should be addressed: Dept. of Pediatrics, NRC5 School of Medicine, Oregon Health Sciences University, Portland, OR 97201-3042. Tel.: 503-494-1930; Fax: 503-494-1933.

[1] The abbreviations used are: IGFBP, insulin-like growth factor-binding protein; rh, recombinant human; PAGE, polyacrylamide gel electrophoresis; WLB, Western ligand blots; ER, estrogen receptor.

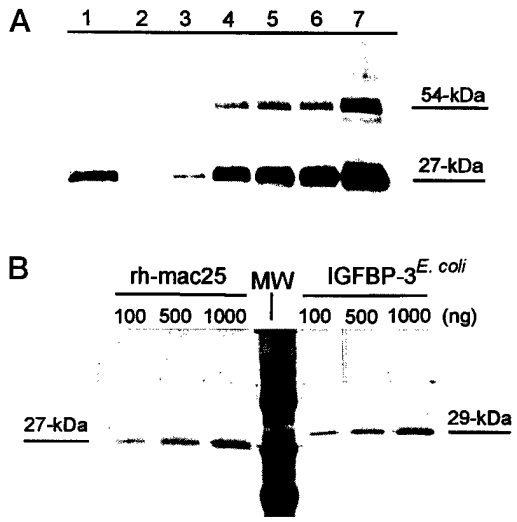

FIG. 1. **Purification of FLAG epitope-tagged mac25.** *A*, purification and Western immunoblotting of baculovirus recombinant mac25/FLAG using anti-M2 monoclonal antibody. Lanes represent 10 μl of samples; media alone from the HI-5 infected cells (*lane 1*), flow-through from the M-2 immunoaffinity column (*lane 2*), eluted fractions of rh-mac25 by FLAG peptides (*lanes 3–6*), or the purified rh-mac25 after pooling and concentration (*lane 7*). *B*, silver staining of the purified rh-mac25 and nonglycosylated IGFBP-3$^{E.\ coli}$.

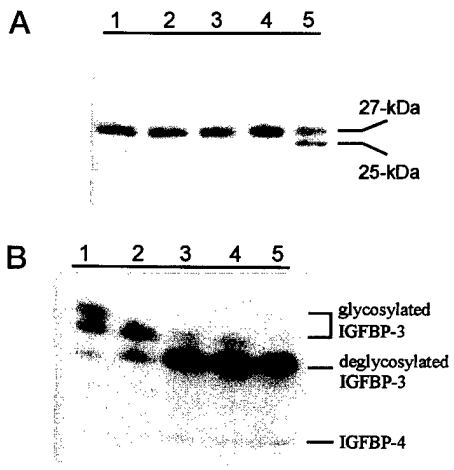

FIG. 2. **Deglycosylation of rh-mac25 and normal human serum IGFBPs with Endo F treatment.** Western immunoblot of rh-mac25 with anti-M2 monoclonal antibody (*A*) and Western ligand blot of normal human serum IGFBPs with ^{125}I-IGF-I (*B*) after treatment with various concentrations of Endo F at 37 °C for 3 h. Lanes represent 40 ng of rh-mac25 alone or 2 μl of human serum alone (*lane 1*) or after Endo F treatment (120 milliunits of Endo-F, *lane 2*; 240 milliunits, *lane 3*; 360 milliunits, *lane 4*; 480 milliunits, *lane 5*).

mac25-pFASTBAC1 construct was transfected into Sf9 insect cells and positive viral recombinants were isolated, using the vendor's protocols. Western immunoblots were performed with the FLAG sequence specific anti-M2 antibody (Eastman Kodak).

Protein Purification and Sequencing—Large scale protein purification was begun by infecting 10^8 HI-5 insect cells at a multiplicity of infection of 3 at 27 °C for 3 days. The media from the infected cells were collected and concentrated, and the resultants were bound to an anti-M2 antibody affinity column overnight at 4 °C. The column was washed three times with 5 ml of HBS (20 mM Hepes, pH 7.8, 150 mM NaCl), and the protein was eluted with four 1-ml washes with HBS containing FLAG peptide, 0.5 μg/μl. The purified protein was subjected to SDS-PAGE in a 12% gel (10), and stained with Coomasie Blue or transferred to nitrocellulose for immunodetection. Eluted fractions from an anti-M2 antibody affinity column were pooled, concentrated, and quantitated by comparison with known amounts of IGFBP-3$^{E.\ coli}$ after silver staining. The NH_2-terminal sequence of mac25 (10 μg) was determined (Biotechnology Laboratory in the Institute of Molecular Biology, University of Oregon) by Edman degradation using a 470A gas-phase protein Sequencer equipped with a 120A phenylthiohydantoin analyzer (Applied Biosystems, Foster City, CA).

Affinity Cross-linking—FLAG-tagged-mac25 and IGFBP-3$^{E.\ coli}$ were incubated with ^{125}I-IGF-I or -IGF-II (50,000 cpm) in the presence or absence of unlabeled peptides at the concentrations indicated in the text and figures, overnight at 4 °C. After cross-linking with disuccinimidyl suberate, samples were subjected to SDS-PAGE and radioautography (10). Bands were quantified by densitometry using the area under the curve, as calculated by an LKB densitometer.

Western Ligand Blots (WLB)—Unreduced samples of FLAG-tagged-mac25 and IGFBP-3$^{E.\ coli}$ were subjected to SDS-PAGE and electroblotted onto nitrocellulose filters, which were incubated overnight with 1.5×10^6 cpm of ^{125}I-IGF-I or -IGF-II, washed, dried, and exposed to film. Similarly, WLB were prepared by nondenaturing PAGE, *i.e.* without SDS, in stacking (pH 7.4) or resolving (pH 8.8) gels (17) for assessment of IGF binding.

Glycosylation Studies—Proteins were deglycosylated with endoglycosidase F (Endo F) (9). As a positive control, acid-chromatographed normal human serum IGFBP fractions were prepared (18), with an IGFBP-3 concentration of 3.5 ng/μl, assessed by IGFBP-3 radioimmunoassay (18). Forty ng of FLAG-tagged mac25 and 2 μl of acid-chromatographed normal human serum IGFBP fraction, containing 7 ng of glycosylated IGFBP-3, were treated with 120–480 milliunits of Endo F as described previously (9). Subsequent Western immunoblots or ligand blots were performed as described above.

Northern Blot Analysis—Blots of 2 μg of poly(A)$^+$ RNA from normal human tissues, that had been subjected to electrophoresis in 1.5% formaldehyde-agarose gel before transfer to nylon membranes, were purchased (Clontech). Total RNA from a variety of normal and cancer cell lines was isolated by standard methods, using guanidine thiocyanate (16). ^{32}P-Labeled antisense cRNA probes for mac25, transcribed from the plasmid constructs, were used. Blots were hybridized and washed at high stringency as described previously (16).

RESULTS

Construction and Expression of FLAG Epitope-tagged mac25—Fig. 1*A* is an immunoblot of the fractions collected during purification of FLAG-tagged mac25, and the purified concentrated protein on nonreducing 12% SDS-PAGE. The major antibody-specific protein is 27 kDa, with a minor higher molecular weight antibody-specific protein of ~54 kDa (presumably dimers of the mac25 protein). Analysis of the purified recombinant human (rh)-mac25 protein on a nonreducing 12% SDS-PAGE and subsequent silver staining (Fig. 1*B*), show a protein of approximately 99% purity and a molecular weight of 27,000. The NH_2-terminal sequence of the purified rh-mac25 was **SSSDTCGPCE**, indicating that human mac25 contains 26 residues of signal peptide sequence and 251 amino acids of the mature protein, which is 18–22% identical to other mature IGFBPs (most identical to IGFBP-3).

Since the deduced amino acid sequence analysis revealed that mac25 contains one potential *N*-glycosylation site located at amino acid 171 (Asn-Val-Thr), we treated mac25 with various concentrations of Endo F (120–480 milliunits) to cleave the *N*-glycosylated carbohydrates. As shown in Fig. 2*A*, the size of mac25 was reduced to approximately 25 kDa following treatment with 480 milliunits of Endo-F, indicating that the secreted rh-mac25 is a glycosylated protein with 2 kDa of *N*-linked sugars and a 25-kDa core. Parallel treatment of human serum IGFBP-3 with Endo F reduced the 41- and 39-kDa glycosylated species to a 29-kDa core protein (Fig. 2*B*).

Characterization of mac25 as IGFBP-7—To test whether mac25 is capable of binding to IGF peptides, we performed WLB and IGF affinity cross-linking. As shown in Fig. 3*A*, when we employed WLB under denaturing conditions with ^{125}I-IGF-I (*upper panel*) or ^{125}I-IGF-II (*lower panel*), rh-mac25 was not detected, at concentrations of rh-mac25 ranging from 1.5 to 150 nM, although 5 nM rhIGFBP-3$^{E.\ coli}$ was detected as a 29-kDa band with either ^{125}I-IGF-I or ^{125}I-IGF-II. However, when rh-mac25 was electrophoresed by nondenaturing PAGE, rh-mac25 was faintly detected at a concentration of 30 nM and clearly

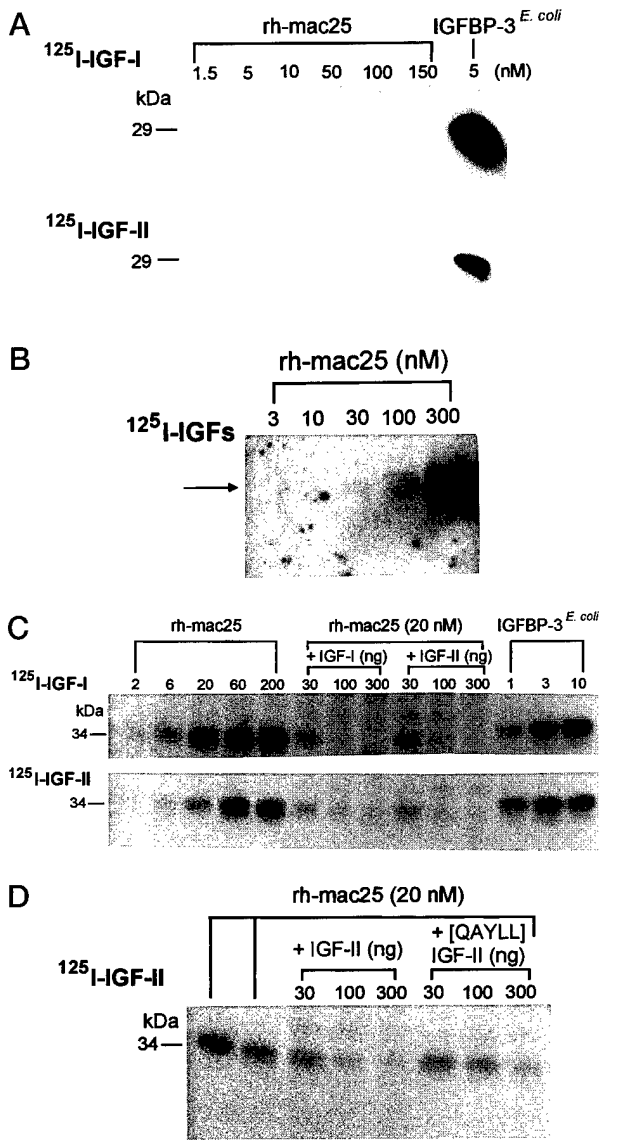

FIG. 3. **WLBs and affinity cross-linking of rh-mac25.** WLBs of various concentrations of rh-mac25 and 5 nM IGFBP-$3^{E.\ coli}$ with ^{125}I-IGFs after denaturing SDS-PAGE (*A*) or after nondenaturing PAGE (*B*). *C*, autoradiogram of ^{125}I-IGF-I (*upper panel*) or ^{125}I-IGF-II (*lower panel*) cross-linked to rh-mac25 or IGFBP-$3^{E.\ coli}$. Radiolabeled ligands (0.5×10^5 cpm) were incubated with 2–200 nM rh-mac25 alone or with 20 nM rh-mac25 in the presence of unlabeled IGF-I or IGF-II. To compare the binding affinity of rh-mac25 for IGFs, 1–10 nM IGFBP-$3^{E.\ coli}$ were cross-linked with the same amount of radiolabeled ligands. *D*, autoradiogram of ^{125}I-IGF-II cross-linked to 20 nM rh-mac25 in the absence or presence of different concentrations of unlabeled IGF-II or [QAYLL]IGF-II.

identified at 300 nM (Fig. 3*B*).

Alternatively, when affinity cross-linking, another method for testing binding ability, was performed with ^{125}I-IGF-I or ^{125}I-IGF-II and concentrations of rh-mac25 similar to those used in WLB, an approximately 34-kDa band was detected on the SDS-PAGE gel (Fig. 3, *C* and *D*), indicating the 27-kDa rh-mac25 bound to 7-kDa ^{125}I-IGF-I (Fig. 3*C*, *upper panel*) or ^{125}I-IGF-II (Fig. 3*C*, *lower panel*, and *D*). Binding of rh-mac25 to IGFs is readily detectable by affinity cross-linking at a mac25 concentration as low as 2 nM. Furthermore, the data indicate that rh-mac25 binding to IGFs is specific, as shown by competition with unlabeled IGF-I and IGF-II, but not with [QAYLL]IGF-II, which has approximately 100-fold less affinity for IGFBPs. We estimated that the affinity of rh-mac25 is at least 5–6-fold lower for IGF-I and 20–25-fold lower for IGF-II than those of rhIGFBP-$3^{E.\ coli}$ (shown as an approximately 36-kDa band), after determining band densities by densitometer.

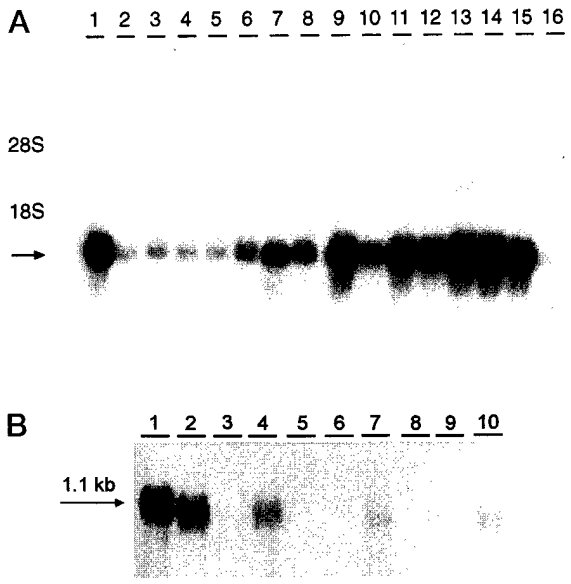

FIG. 4. **Northern blot analysis of IGFBP-7 in human normal tissue and cancer cell lines.** *A*, normal tissue: human multiple tissue blots were probed with mac-25 cRNA and exposed to BIOMAX film for 2 h at −80 °C. *Lanes 1–16* are: *1*, heart; *2*, brain; *3*, placenta; *4*, lung; *5*, liver; *6*, skeletal muscle; *7*, kidney; *8*, pancreas; *9*, spleen; *10*, thymus; *11*, prostate; *12*, testis; *13*, ovary; *14*, small intestine; *15*, colon; *16*, peripheral blood leukocyte. *B*, cancer cell lines: 10 μg of total RNA was used from each cell line. Blots were exposed to BIOMAX film for 20 h at −80 °C. *Lane 1*, Hs578T (ER-negative breast cancer); *2*, MDA-MB-231 (ER-negative breast cancer); *3*, MCF-7 (ER-positive breast cancer); *4*, MCF-10F (normal mammary epithelial cells); *5*, ZR75–1 (ER-positive breast cancer); *6*, WiDr (colon cancer); *7*, prostate (normal); *8*, PC3 (prostate cancer); *9*, H417 (small cell lung carcinoma); *10*, A175 (glioblastoma).

Expression of IGFBP-7 mRNA in Normal Human Tissues and Cancer Cells—We next investigated the distribution of IGFBP-7 in normal human tissues and human cancer cells, using Northern blot analysis. As shown in Fig. 4*A*, the 1.1-kilobase IGFBP-7 mRNA was detected in a broad spectrum of normal tissues. In particular, high expression of IGFBP-7 was observed in small intestine, colon, ovary, prostate, testes, spleen, heart, kidney, and pancreas. Interestingly, expression of IGFBP-7 mRNA was reduced in ER-positive breast cancer and cancer cells from prostate, colon, and lung, although ER-negative breast cancer cells and glioblastoma cells showed relatively high expression (Fig. 4*B*).

DISCUSSION

IGFBPs bind to IGF peptides with high affinity, ranging from 10^{-11} to 10^{-9} M and regulate the biological activities of the IGFs. Amino acid sequence analysis has revealed that relatively high similarity (50–60%) exists among human IGFBPs (1). The most striking feature is a cluster of 18 conserved cysteines found in all IGFBPs except IGFBP-6, which contains 16. Twelve are in the NH_2-terminal one-third of the molecule (10 in IGFBP-6) and the remaining six are in the COOH-terminal third of the protein. These terminal regions are highly homologous among the IGFBPs and are speculated to contribute to binding of IGFs. In regard to this sequence homology and striking conservation of cysteines among the IGFBPs, mac25 meets structural criteria as a new member of the IGFBP family by the fact that mac25 contains critical conserved sequences, including the IGFBP motif (GCGCCXXC) in the NH_2 terminus,

as well as 11 conserved cysteines in the NH_2 terminus and, possibly, 1 at the COOH terminus. Indeed, our affinity cross-linking data showed that mac25 specifically binds IGF-I and IGF-II, indicating that mac25 is a bona fide IGFBP, and can be properly called IGFBP-7. Specificity of the IGFBP-7 binding to IGF was further demonstrated by the relatively low affinity for [QAYLL]IGF-II, an IGF-II analog whose affinity for IGFBPs is 100-fold less than that of native IGF-II.

Compared with IGFBP-3, the affinity of IGFBP-7 for IGFs was assessed by competitive affinity cross-linking to be 5–25-fold lower, which might be attributed to a lack of conserved sequences at the COOH terminus, particularly of the 6 cysteines, of which only one may be conserved. The COOH terminus of the IGFBPs clearly contributes to the structural configuration required for IGF binding and may even be capable of independent binding of IGF peptides.[2] The failure to demonstrate IGFBP-7 binding of IGF by conventional Western ligand blotting methods may be attributable to the loss of structural integrity under denaturing conditions, since binding was observed when blotting was performed with non-denaturing gels. Alternatively, the affinity of IGFBP-7 for IGF peptides may be overestimated by cross-linking and may, in fact, be 2 or even 3 orders of magnitude lower than that of IGFBP-3. Similar problems have been observed in studies of proteolytic fragments of IGFBP-3, which bind poorly by Western ligand blot, but are readily identified by cross-linking (19). Nevertheless, even if the affinity of IGFBP-7 proves to be 100-fold lower than that of IGFBP-3, it would still be in a range capable of modulating the interaction of IGFs with their receptors, as is the case with other IGFBPs.

Recent studies from our laboratory have demonstrated that IGFBP-3, the major IGFBP species in the circulation, has a novel growth inhibitory action mediated through its own receptor (IGF-independent action), in addition to regulating IGF access to IGF receptors (IGF-dependent action) in human breast cancer cells (9, 10). Furthermore, transcriptional regulation of IGFBP-3 expression provides a mechanism for both TGF-β2 and all-*trans*-retinoic acid (all-*trans*-RA) inhibition of breast cancer cell growth (11, 12). Previous studies have reported that expression of mac25 mRNA was elevated in senescent human mammary epithelial cells, while apparently down-regulated in mammary carcinoma cell lines (13, 14). Moreover, mac25 mRNA was up-regulated in normal, growing mammary epithelial cells by all-*trans*-RA, suggesting that mac25 may be a downstream effector of the RA-induced senescence pathway (14). Taken together with our data showing reduced levels of mac25 (IGFBP-7) mRNA in cancer cells from breast, prostate, colon and lung, it is tempting to speculate that mac25 (IGFBP-7) functions not only as an IGF binding protein, but also as a direct growth-suppressing factor, with an IGF-independent action similar to that of IGFBP-3 (9–12). Additionally, we propose that IGFBPs 1–7 will prove to be members of a superfamily of growth-regulating factors that share structural and sequence homology and which may modulate growth by IGF-dependent and/or IGF-independent mechanisms. Further assessment of IGF binding affinity and the mechanism(s) for the growth-suppressing actions of IGFBP-7 is currently underway.

[2] Y. Oh, S. R. Nagalla, Y. Yamanaka, H.-S. Kim, E. Wilson, and R. G. Rosenfeld, unpublished data.

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GENERATION AND CHARACTERIZATION OF AN IGFBP-7 ANTIBODY: IDENTIFICATION OF 31kD IGFBP-7 IN HUMAN BIOLOGICAL FLUIDS AND Hs578T HUMAN BREAST CANCER CONDITIONED MEDIA

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ABSTRACT

The cDNA encoding mac25 (IGFBP-7) was first derived from mRNA isolated from leptomeningial and senescent human mammary epithelial cells (1,2) . The open reading frame was shown to predict a protein with homology to the amino terminus of the IGF binding proteins, (IGFBP)1-6 . Studies in our laboratory have shown that baculovirus generated mac25 binds IGF-I and-II in a specific manner, leading to the renaming of mac25 as IGFBP-7 (3). Further studies at the cellular level, to identify the involvement of IGFBP-7 in IGF regulation and cell growth, require a specific antibody against the protein, which has yet to be identified in either cultured cells or *in vivo*. We have now generated three polyclonal antibodies against the purified baculovirus peptide and, by western immunoblots and immunoprecipitation, demonstrated the existence of a specific 31,000 dalton protein. It is a secreted protein, and can be identified in the conditioned media of Hs578T breast cancer cells , as well as in normal human urine, cerebrospinal fluid and amniotic fluid. Subsequent studies with these antibodies should help elucidate the physiological role(s) of this protein.

INTRODUCTION

The cDNA encoding mac25 was first derived from mRNA from leptomeningial cells, and, subsequently, was observed through differential display in human senescent mammary epithelial cells(1,2). The predicted mac25-encoded protein was shown to display homology to the six insulin-like growth factors binding proteins (IGFBP), especially at the N-terminus, where eleven out of the customary twelve conserved cysteines were identified (1,3-,7). This homology led to the hypothesis that mac25 acts as a new IGFBP, with a role in regulation of cellular replication and senescence. Our laboratory has recently shown that baculovirus generated mac25 protein binds both IGF-I and-II in a specific manner, although with lower affinity than observed with IGFBP-1 through-6, resulting in the renaming of mac25 as IGFBP-7 (3). Although we demonstrated the presence of mRNA for IGFBP-7 in a wide variety of tissues, to date the protein has not been demonstrated to exist *in vivo*. To study the *in vivo* distribution and function of IGFBP-7, we have now produced polyclonal antibodies against recombinant IGFBP-7^{bac} and have demonstrated the existence of the native protein in human biological fluids, as well as in a human breast carcinoma cell line, Hs578T.

MATERIAL AND METHODS

Tissue Culture and Cell Lines: Hs578T cells were purchased from ATCC and grown in DMEM plus 10% FCS at 37C in 5% CO_2. All tissue culture media and components were purchased from Lifetechnologies (Gaithersburg, MD). HIGH-5 cells were obtained from Invitrogen Inc. (San Diego, CA) and were maintained in ExCell 405 Media (JRH Biosciences, Lenexa, KS) at 27C. The wild type baculovirus (AcNPv) was obtained from Invitrogen, Inc. (San Diego, CA).

Sources of IGFBP-1-6: HPLC purified hIGFBP-1 from human amniotic fluid was kindly provided by Dr. D. R . Powell (Baylor College of Medicine, Houston, TX). Recombinant human IGFBP-3, a nonglycosylated 29kDa core protein expressed in Escherichia coli cells, was the generous gift of Celtrix, Inc. (Santa Clara, CA). Recombinant human IGFBP-2, -4, -5 and -6 were purchased from Austral Biologicals (San Ramon, CA).

Plasmid Constructs and Baculovirus Recombinants: The cDNA for IGFBP-7 (mac25) was cloned and baculovirus recombinants made as described in Ref. 3. Briefly, the cDNA was cloned from an Hs578T cDNA library and sequenced; the FLAG tag epitope (DYKD-DDDK) was added to the C-terminus by PCR and then subcloned into a baculovirus recombination vector, and recombinant viruses were produced as described in Lifetechnologies Bac-to-Bac protocol (Gaithersburg, MD).

Protein Purification and Immunization of Rabbits: The baculovirus expressed IGFBP-7:FLAG (Ac:$IGFBP^{flag}$) was purified as described in Ref. 3. Briefly, protein was overexpressed in Ac:IGFBP-7^{flag} infected HIGH-5 insect cells. Media collected by centrifugation of cells and dialyzed in 20mM Hepes pH 7.8, 150mM NaCl, was passed over a Mono Q Ion exchange column (Pharmacia Biotech, Piscataway, NJ) and proteins eluted with a linear salt gradient of 50mM NaCl to 500mM NaCl in 20mM Hepes pH 7.4. Mono Q fractions were further purified over the FLAG sequence specific anti-M2 Antibody affinity column (Eastman Kodak Co., New Haven, CT). Three New Zealand White female rabbits were injected subcutaneously with 50μg of purified IGFBP-7^{bac} mixed with an equal volume of Freund's complete adjuvant. Subsequent boosts were done subcutaneously with 15-25μg of purified protein and Freund's incomplete adjuvant at one month intervals. After the second boost, three rabbits (b1, b2, and,b3) were bled and two rabbits were exsanguinated and sera collected from total blood. The sera from rabbit b1 was used in subsequent experiments.

Western Immunoblotting with anti-IGFBP-7 Antibody: Proteins were separated on a non-reducing 12% SDS:PAGE and transferred to nitrocellulose (10). The membranes were blocked with 5% nonfat dry milk: TBS-T (20mM Tris-Cl pH 7.6, 150mM NaCl, 0.1% Tween-

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20) for one hour at room temperature. The membranes were rinsed three times with TBS-T and incubated in a 1:5000 dilution of anti-IGFBP-7b1 antibody in TBS-T for one hour at room temperature. The membranes were rinsed in TBS-T as before and then incubated in a 1:3000 dilution of anti-rabbit IgG conjugated to horse radish peroxidase (Amersham, Arlington Heights, IL) for one hour at room temperature. The membranes were then washed two times, for 15 minutes each, in TBS-T and the immunoreactive proteins detected by chemiluminescence, using the Renaissance Detection Kit (Dupont/NEN, Wilmington, DE).

Immunoprecipitation of ^{35}S metabolically labeled Hs578T Proteins: Confluent monolayers of Hs578T cells were rinsed three times with DMEM (without methionine and cysteine), incubated in DMEM (without methionine and cysteine) with 250μCi/ml of EXPRE^{35}S^{35}S Label (DuPont/NEN, Wilmington, DE) for 16 hour at 37C in 5% CO_2, and the conditioned media collected. Immunoprecipitations were done with 1.25ml of conditioned media and 5μl of either preimmune sera, anti-IGFBP-7b1, or anti-IGFBP-3g1 antibody (8), followed by incubation for one hour on ice. Protein-A Sepharose beads that had been equilibrated in 20mM Hepes, pH 7.8, 150mM NaCl, 1% Triton-X100, 1% Sodium deoxycholate were added, to bind the antigen/antibody complex, and incubated for one hour at 4C, rocking end over end. The beads were centrifuged and washed two times in the above buffer. The proteins were eluted by boiling for three minutes in non-reducing SDS sample buffer and then subjected to a non-reducing 10% SDS:PAGE followed by autoradiography.

Immunoprecipitations and western immunoblotting of biological fluids and conditioned media: Anti-IGFBP-7b1 antibody was covalently crosslinked using dimethyl pimelimdate (Sigma Chemical Co., St. Louis, MO) to Protein A Sepharose (9) and used to immunoprecipitate normal human sera, normal human urine, human amniotic fluid, human cerebrospinal fluid (CSF), and conditioned media from Hs578T. The immunoprecipitation products were then analyzed by western immunoblot, using the anti-IGFBP-7b1 antibody, as described above.

RESULTS

Production of antibody to IGFBP-7^{bac}: All three rabbits developed antibodies to IGFBP-7, Figures 1 and 2 show that the polyclonal antibody anti-IGFBP-7b1, produced against purified baculovirus expressed IGFBP-7, can detect both recombinant and native protein. Figure 1 shows a western immunoblot of conditioned media from HIGH-5 cells that were either infected with wild type baculovirus (AcNPV) or IGFBP-7^{bac}. The antibody specifically reacts with the 27kDa recombinant protein and does not cross-react with endogenous baculovirus proteins. Figure 2 depicts an immunoprecipitation of ^{35}S metabolically labeled conditioned media from Hs578T cells with either pre-immune sera from the rabbits before immunization, anti-IGFBP-7b1 or anti-IGFBP-3g1. There is a specific immunoreactive protein of approximately

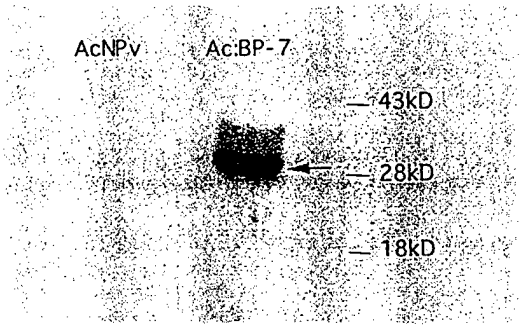

Figure 1. Western Immunoblot using anti-IGFBP-7-b1 against conditioned media from HIGH-5 cells infected with AcNPv (wildtype baculovirus) or IGFBP-7^{bac}. Twenty microliters from 2.0ml of media from a 35mm dish for each sample were analyzed.

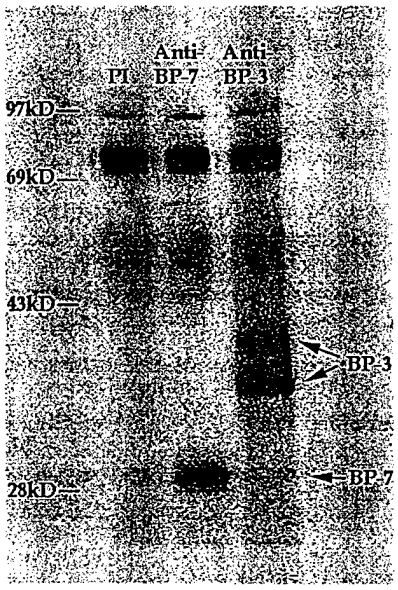

Figure 2. Immunoprecipitation of ^{35}S Methionine/Cysteine labeled Hs578T breast carcinoma cell conditioned media (CM) with rabbit pre-immune serum (PI), anti-IGFBP-7-b1(anti-BP-7), or anti-IGFBP-3-g1 (anti-BP-3). Each lane represents 1.35ml of ^{35}S labeled CM immunoprecipitated with 5μl of the indicated serum or antibody

31kDa that is immunoprecipitated with anti-IGFBP-7b1, but not by preimmune sera or anti-IGFBP-3g1. Immunoblots performed with anti-IGFBP-7b1 and pure IGFBP-1 through -6 were negative, even at IGFBP concentrations as high as 200ng (data not shown)

Identification of native IGFBP-7 from other sources: Immunoprecipitations were preformed using anti-IGFBP-7b1 covalently crosslinked to Protein A Sepharose, to detect the presence of IGFBP-7 in normal human sera, amniotic fluid, CSF, and urine. Hs578T CM and Protein A Sepharose beads alone were used as positive and negative controls, respectively. The immunoprecipitated products

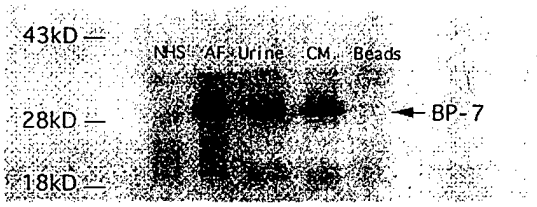

Figure 3. Immunoprecipitations and western immunoblotting of biological fluids or conditioned media from Hs578T cells using anti-IGFBP-7-b1 antibody. Immunoprecipitations were done on 20μl of normal human sera, 200μl of urine, and 1ml of amniotic fluid (A.F.). CM from Hs578T cells, and Protein A Sepharose beads alone served as positive and negative controls, respectively

were then analyzed by western immunoblot using anti-IGFBP-7-b1, as described in the Materials and Methods. Figure 3 shows that there is a similarly sized 31kDa immunoreactive protein in urine and amniotic fluid, and, after prolonged exposure, a faint 31kDa band could be seen in normal sera (data not shown). A 31kDa IGFBP-7 immunoreactive band could also be identified after immunoprecipitations of human CSF (data not shown). Further studies with endoglycosidase F revealed that the 31kDa native IGFBP-7 is a glycosylated protein with 4 kDa of N-linked sugars and a 27kDa core protein (data not shown).

DISCUSSION

Our laboratory has recently demonstrated through competitive affinity cross-linking studies with IGF-I and -II, and native western ligand blotting with ^{125}I-IGF-I and -II, that mac25 is a new member of the IGFBP family, IGFBP-7(3). In conjunction with these studies, we have shown IGFBP-7 mRNA to be widely distributed in many normal human tissues.

Previous reports have shown that IGFBP-7 mRNA is up-regulated by treatment with all-*trans*-retinoic acid in normal human mammary epithelial cells (2). Given the relatively low affinity of IGFBP-7 for IGF-I and -II, we hypothesize that IGFBP-7 may have an IGF-independent role in the cellular scenecence and growth suppression pathways. In the present study, we have now generated and characterized polyclonal antibodies to recombinant human IGFBP-7. Our antibodies specifically recognizes recombinant IGFBP-7 and native IGFBP-7 in breast cancer cells and some biological fluids, such as urine, CSF and amniotic fluid. The antibodies are immunoreactive for the native protein, as shown by immunoprecipitations, and to the denatured protein, as demonstrated by western immunoblotting. To our knowledge, this is the first report that IGFBP-7 protein is produced by cultured cells, and is present in normal biological fluids. Further *in vivo* and *in vitro* studies using the recombinant peptide and appropriate antibodies will facilitate assessment of the physiological significance of IGFBP-7.

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SYNTHESIS OF IGFBP-3 FRAGMENTS IN A BACULOVIRUS SYSTEM AND CHARACTERIZATION OF MONOCLONAL ANTI -IGFBP-3 ANTIBODIES.

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ABSTRACT

IGFBPs play an important role in IGF biological actions by modulating IGF binding to its receptors. The major IGFBP in serum is IGFBP-3, which transports 70-90% of the circulating IGFs. In target cell systems, it sequesters IGFs and inhibits their hormonal actions, but may potentiate IGF activity or exert IGF-independent effects under specific conditions. IGFBP-3 can be modified by IGFBP-3 proteases, which degrade it into smaller fragments. IGFBP-3 fragments generated by proteolysis have reduced affinity for IGFs, thereby modifying IGF action. To study IGFBP-3 fragments *in vivo* and *in vitro*, we constructed six different IGFBP-3 fragments by use of a baculovirus expression system and generated 8 different monoclonal IGFBP-3 antibodies. Based on the known cleavage sites of IGFBP-3 for PSA, MMPs, and the predicted plasmin cleavage sites, we expressed a N-terminal IGFBP-3^{1-97} fragment and a C-terminal IGFBP-3^{98-264} fragment. By stepwise truncation from the C-terminal end, we created IGFBP-3^{98-232} , IGFBP-3^{98-206} ,IGFBP-3^{98-179} , and IGFBP-3^{98-159}. A strong recognition of the C-terminus and the intermediate parts of IGFBP-3 by six antibodies was found. Four of these mAbs were able to recognize the intermediate fragment alone. Two mAbs were found to immunoreact only with the N-terminal IGFBP-3 fragment and two additional mAbs recognized the N- as well as the C-terminal parts and lacked immunoreactivity for the intermediate part of IGFBP-3. The 15 kDa IGFBP-3 fragment resulting from plasmin digestion was found to only react with N-terminal antibodies, while the 29 kDa fragment in pregnancy serum reacted with both N- and C-terminal antibodies. Thus, these mAbs will be useful tools to determine whether IGFBP-3 fragments found in vivo derive from either the N- or C-terminal domains of IGFBP-3.

INTRODUCTION

IGFBP-3, the major carrier of IGF peptides, is a target for a variety of different proteases *in vivo* and *in vitro* (1,2,4-6) The resulting IGFBP-3 fragments have reduced affinity for IGFs, thereby modifying their cellular action in target systems (3). Recent reports have described IGF-independent bioactivity of some IGFBP-3 fragments (7,8). Although some studies describe N-terminal sequences of IGFBP-3 fragments derived by different proteases *in vitro*, the exact sequences of *in vivo* IGFBP-3 fragments and their biological function remain unclear. It has been shown that the plasminogen/ plasmin system plays an important role in the proteolysis of IGFBP-3 and is also part of pregnancy-associated protease activity (4,7,8). Based on the information from plasmin-, PSA- and MMP- induced proteolysis of IGFBP-3, we have expressed six different IGFBP-3 fragments in a baculovirus system. We also generated 8 different monoclonal antibodies (mAb), raised against human recombinant IGFBP-$3^{E.coli}$. In this study, we have employed these mAbs to characterize synthetic and plasmin-generated IGFBP-3 fragments.

MATERIAL AND METHODS

plasmid constructs and baculovirus expression: cDNAs for six IGFBP-3 fragments (construct B-G, fig. 1A), as well as the full length IGFBP-3 (construct A) were subcloned into pFASTBAC HTa baculovirus expression vector (Gibco, Gaithersburg, MD). All constructs were made by PCR, using human IGFBP-3 cDNA as template and oligonucleotide primers carrying either an unique in frame NarI restriction site (5' primer) or a stop codon and a XbaI site (3' primer) for subcloning the resulting PCR product into the baculovirus expression vector (table 1).

Table 1

primer sequences used for construction of six different IGFBP-3 fragments in pFASTBAC HTa.

	amino acid	5'primer	3'primer
A	1-264	CGGGCTGGCGCCAGCTC GGGG	CGCTCTAGACTACCTGCTCTG CATGCTG
B	1-97	CGGGCTGGCGCCAGCTC GGGG	CGCTCTAGACTAGCGCAGGC GGCTGACGGC
C	98-264	TCAGGGCGCCTACCTGC TGCCAGCGCCG	CGCTCTAGACTACCTGCTCTG CATGCTG
D	98-232	TCAGGGCGCCTACCTGC TGCCAGCGCCG	CGCTCTAGACTACCGCTTCCT GCCTTTGGAAG
E	98-206	TCAGGGCGCCTACCTGC TGCCAGCGCCG	CGCTCTAGACTACTTGGGAC TCAGCACATTG
F	98-179	TCAGGGCGCCTACCTGC TGCCAGCGCCG	CGCTCTAGACTACCGCTTGG ACTCGGAGGAGAAG
G	98-159	TCAGGGCGCCTACCTGC TGCCAGCGCCG	CGCTCTAGACTAGTAGCGCT GGCTGTCTTTAGC

Because the protein processing steps in insect cells are similar to those in mammals, the signal peptide sequence of cDNA in the full length IGFBP-3, as well as in the N-terminal fragment, was deleted, to prevent the loss of the additional amino acids from the pFASTBAC HTa vector as described below. All constructs were confirmed by sequencing. Recombinant viruses were produced according to the manufacturer's protocol (Gibco, Gaithersburg, MD). All expressed proteins contain, on their N-terminal end, 23 additional amino acids, including 6 Histidines and a rTEV protease cleavage site. Proteins were overexpressed in SF-9 insect cells. Three days after infection with the recombinant

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virus, the cells were harvested and lysed in lysis buffer (100 mM Hepes pH 7.4, 50 mM NaCl containing Pepstatin, Leupeptin and Aprotinin in a final concentration of 0.1 mg/ml) by sonication on ice. Centrifugation at 50.000 x g for 30 min was performed to remove all insoluble material.

generation of monoclonal anti IGFBP-3 antibodies: Mouse monoclonal antibodies were raised against recombinant, non-glycosylated human IGFBP-$3^{E.coli}$ (Celtrix, Santa Clara, CA). BALB/c mice were immunized subcutaneously with IGFBP-3 antigen. The splenocytes from immunized mice were fused with myeloma cells by the polyethylene glycol method. The viable hybridomas were selected, screened and propagated. The supernatants from clones were selected and screened against IGFBP-3 by ELISA technique. The selected hybridomas were cloned by the limiting dilution method. Eight monoclonal mAbs for IGFBP-3 were selected and propagated in culture, and cells were injected into BALB/c mice for ascites production. Anti-IGFBP-3 IgG antibodies were purified on protein A column and tested on western immunoblots and in immunoassays.

western immunoblotting: Proteins were separated on a nonreducing 15 % SDS-PAGE (National Diagnostics, Atlanta, GO) and transferred to Hybond ECL nitrocellulose membrane (Amersham). Immunodetection was performed as previously described (9). We used a 1:3000 dilution of polyclonal αIGFBP-3ng1 antibody (10), 1:500 of mouse acites or 1:3000 of the purified monoclonal antibodies.

proteolysis of IGFBP-3: 0.2 µg of recombinant human IGFBP-$3^{E.coli}$ were digested with 44 µg plasmin (Fluka, Ronkonkoma, NY) for 150 min at 37°C in Tris buffer (0.02 M tris ph 7,4; 0,15 M NaCl). After digestion, the reaction was stopped with SDS sample buffer and loaded immediately on a 15 % SDS- PAGE.

RESULTS

Figure 1A shows the different IGFBP-3 constructs expressed in a baculovirus system. The A construct corresponds to the full length IGFBP-3 sequence. Fragment B (IGFBP-3^{1-97}) contains the predicted major IGF binding site in the N-terminal region, which is highly conserved among the IGFBPs. Fragment C (IGFBP-3^{98-264}) includes the intermediate and C-terminal parts of IGFBP-3. Four additional fragments (D-G) have the same N-terminal part as fragment C. They have been stepwise truncated on their C-terminal end. The D fragment (IGFBP-3^{98-232}) lacks the C-terminal region, which is highly conserved among the IGFBPs and considered as a possible second part of the IGF-binding site. Fragment E (IGFBP-3^{98-206}) lacks the major heparin binding domain; F-fragment (IGFBP-3^{98-179}) lacks part of the predicted minor heparin binding motif and a N-glycosylation site, and fragment G (IGFBP-3^{98-159}) is more truncated at the C-terminus. Figure 1B shows a western immunoblot of 20 µl cell lysates from SF9 cells transfected with Bacmid DNA containing the different IGFBP-3 fragment cDNAs. All fragments were successfully expressed and could be detected by αIGFBP-3ng1 antibody.

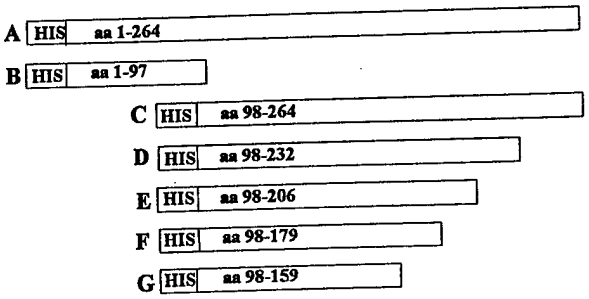

Figure 1A: construction of IGFBP-3 fragments.

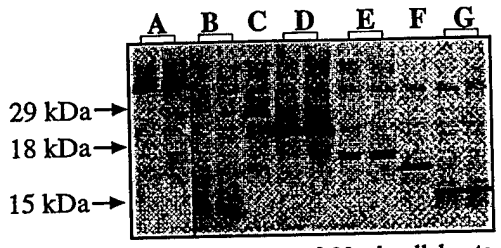

Figure 1B: Western immunoblot of 20 ul cell lysate from IGFBP-3 fragment expressing SF-9 cells, using αIGFBP-3ng1 antibody.

Cell lysates from SF9 cells expressing the different IGFBP-3 fragments were then used to map the binding epitopes of eight different mAbs. Ten µl cell lysates of infected SF9 cells with each IGFBP-3 fragment were loaded on a SDS- PAGE. Western immunoblots with the different monoclonal anti-IGFBP-3 antibodies were performed (Fig. 2A). All mAbs recognized full-length IGFBP-3^{bac} (construct A). Figure 2B is the summary of the recognition pattern of the different mAbs for the IGFBP-3 fragments. The N-terminal fragment B (IGFBP-3^{1-97}) was recognized by mAb3, 5, 6 and 8. The C-terminal fragment C (IGFBP-3^{98-264}) was strongly recognized by mAbs1, 2, 4, 5 and 7, as well as more weakly by mAb8. C-terminal truncation of aa 233-264 from the C fragment results in loss of the binding epitope of this fragment for mAb5. Further truncation on the C-terminal end (aa 207-232) prevents the binding of mAb8. The intermediate aa 98-206 are strongly detected by mAb1,2,4 and 7.

We used purified mAb2 and mAb3 to characterize the 29 kDa IGFBP-3 fragment from human pregnancy serum and the major 15 kDa IGFBP-3 fragment resulting from plasmin digestion. Figure 3 shows the western immunoblot of human pregnancy serum and plasmin-digested IGFBP-3. The 29 kDa IGFBP-3 fragment in human pregnancy serum is recognised by both antibodies, whereas the major 15 kDA IGFBP-3 fragment resulting from plasmin digestion is only recognised by the N-terminal antibody, mAb3.

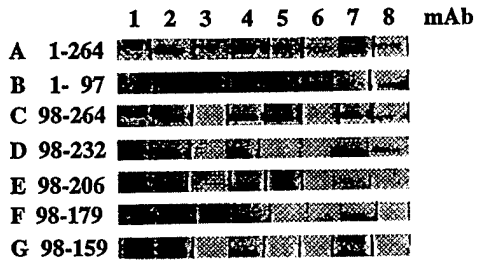

Figure 2A: Western immunoblot of IGFBP-3 fragments, using 8 mAbs

		#1	#2	#3	#4	#5	#6	#7	#8
A	1-264	+	+	+	+	+	+	+	+
B	1-97			+		+	+		+
C	98-264	+	+		+	+		+	+
D	98-232	+	+		+			+	+
E	98-206	+	+		+			+	
F	98-179	+	+		+			+	
G	98-159	+	+		+			+	

figure 2B: recognition pattern of the 8 mAbs.

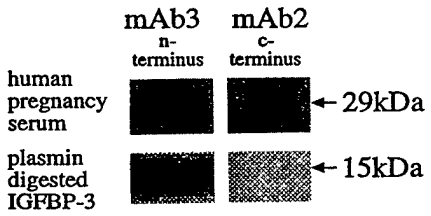

Figure 3: western immunoblot of human pregnancy serum and plasmin digested IGFBP-3, using mAb2 and 3.

DISCUSSION

IGFBP-3 proteases and the resulting IGFBP-3 fragments have been the subject of extensive study since their first detection in human pregnancy serum (1,2). These fragments were initially identified by their failure to bind iodinated IGFs on western ligand blotting, but were recognized by a variety of IGFBP-3 polyclonal antibodies (3). Characterization of the proteolytic fragments has been hampered by difficulty in purifying them from biological fluids. We have employed a baculovirus expression system to generate intact IGFBP-3 and a variety of N-terminal, C-terminal and mid-region fragments of IGFBP-3, and have employed these fragments to characterize a panel of IGFBP-3 mAbs speciffic for different IGFBP-3 epitopes. The pattern of immunoreactivity identified for the 15 kDa IGFBP-3 fragment derived by plasmin digestion demonstrated it to be an N-terminal fragment. This was further confirmed by N-terminal sequence analysis (data not shown). The major 29 kDa glycosylated IGFBP-3 fragment found in human pregnancy serum was recognized by both mAb2 and mAb3, indicating the presence of both the N-terminus and intermediate portions of IGFBP-3. The use of synthetic IGFBP-3 fragments and mAbs specific for discrete IGFBP-3 epitopes should allow characterization of naturally occurring IGFBP-3 fragments, as well as elucidation of IGF-binding, proteoglycan-binding, and cell membrane binding regions of the molecule.

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